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Radioimmunoassay of Dibenzazepines and Dibenzcycloheptanodienes in Body Fluids and Tissues

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Summary: In an attempt to establish a radioimmunoassay (RIA), imipramine and amitriptyline immunogens were prepared; desmethyl derivatives were converted into hemisuccinates, conjugated with bovine serum albumin and used for rabbit immunization.

[^{3}H]Amitriptyline (4.3 TBq/mmol) and [^{3}H]imipramine (2.9 TBq/mmol) were prepared by catalytic dehalogenation or reductive alkylation. Dibenzazepines and dibenzcycloheptanodienes were determined in biological fluids by a direct method without deproteinization (lower detection limit of 0.5 $\mu g \cdot l^{-1}$); using high-yield methods they were extracted from cell membranes.

Assay of tricyclic antidepressants in humans showed that these substances disappear from plasma much earlier than from cell membranes. Dissociation of the antidepressants bound to cell membranes is slow and their plasma concentrations are not influenced by standing for 2 h at 4 °C. During preparing the membranes for binding studies these substances are not removed, and they may affect the results of the binding studies.

Introduction

Radioimmunological determination of tricyclic antidepressants of the groups of dibenzazepines and dibenzcycloheptanodienes in body fluids has so far been reported in about twenty studies (1). The method exhibits a high sensitivity, good reproducibility and easy performance; it requires very small amounts of biological fluids and tissues, a fact often appreciated in toxicology. Its low detection limit is most useful in clinical practice where the therapeutic amount of administered drug results in plasma levels of 10^1-10^2 $\mu g \cdot l^{-1}$. The method is also suitable for pharmacokinetic and pharmacodynamic studies of these drugs.

Current methods of determination of tricyclic antidepressants mostly aim at direct determination of these substances in plasma or in urine. We have developed a simple and sensitive method for dibenzazepine and dibenzcycloheptanodiene determination not only in body fluids but also in biological membranes. The technique was used to determine the levels of tricyclic antidepressants in plasma, blood elements and cell membranes in patients who had been treated with tricyclic antidepressants.

Materials and Methods

Chemicals

Imipramine hydrochloride and desmethylimipramine hydrochloride, Ciba Geigy, Basle, Switzerland.

Amitriptyline hydrochloride and nortriptyline hydrochloride, Lundbeck, Copenhagen–Vally, Denmark.

Active charcoal Norit, Serva, Heidelberg, Germany.

Polyethylene glycol 6000, Merck, Darmstadt, Germany.

Bovine γ-globulin, Institute of Sera and Vaccines, Prague.

Other reagents were obtained from Labora, Brno, Czechoslovakia. All chemicals were of analytical purity. All solvents were purified by distillation.

^3H was obtained from Technabsexport, USSR and stored in the form of uranium tritide, ^{125}I from the Institute of Atomic Energy, Isotope Production and Reactor Centre, Swierk, Poland.

Silica gel TLC sheets (Silufol, UV 252) were obtained from Kavalier, Czechoslovakia. Visking dialysis tubing was from Serva, Heidelberg, Germany.

Solvent systems for chromatography:

S_1 = 2-propanol : benzene (1 + 1)
S_2 = acetone : ammonia (99 + 1)
S_3 = methanol : ammonia (100 + 1.5)
S_4 = ethyl acetate : n-propanol : ammonia (40 + 30 + 3)
S_5 = methanol : ammonia (100 + 2)
S_6 = chloroform : ethanol : ammonia (12 + 8 + 0.1)
S_7 = methanol : ammonia (95 + 5)
S_8 = benzene : methanol : ammonia (90 + 15 + 1)

Human blood was always collected between 7 and 8 a.m. (EDTA 1 g · l^{-1}). Blood cells were separated by centrifugation and the plasma was used for tricyclic antidepressant determination. Erythrocyte membranes were isolated after water-induced haemolysis of blood pellets at 4 °C followed by centrifugation. The membranes were repeatedly washed with water and the protein content was determined using the method of *Lowry* et al. (2).

Immunogens for dibenzazepines and dibenzcycloheptanodienes were prepared using desmethylderivatives of imipramine and amitriptyline. After conversion into hemisuccinates and conjugation with bovine serum albumin immunization of rabbits was carried out according to *Hubbard* et al. (3) and *Brunswick* et al. (4).

Preparation of tritium-labelled ligands

[^3H]Amitriptyline (I)

1) Bromination of amitriptyline

Amitriptyline hydrochloride (0.068 mmol; 21.4 mg) was dissolved in 0.3 ml of ice-cold acetic acid, 0.31 mmol (16 µl) of bromine was added and the reaction mixture was stirred for 2 days at room temperature. [^{3}H]Amitriptyline was prepared by a modification of the reaction procedure according to *Buchman* et al. (5, 6). The solvent and excess bromine were removed by lyophilization and the residue was dissolved in doxane. Then 0.1 ml of trimethylamine was added and the resulting trimethylammonium bromide was removed by centrifugation. One fourth of the raw product was purified by preparative TLC (S_2).

2) Reductive dehalogenation

One fourth of purified brominated amitriptyline in 0.2 ml methanol and 0.3 ml of triethylamine was added to freshly prereduced palladium oxide (6.1 mg) and the reaction mixture was stirred for 1 h with 2 ml carrier-free tritium (total pressure 0.072 MPa). The catalyst and labile activity were removed. Preparative TLC (S_2) yielded a product of a 97% radiochemical purity (TLC — $S_{2,3,4}$) and a specific activity 4.3 TBq/mmol (118 Ci/mmol).

The overall chemical yield was 18%.

[^3H]Desmethylimipramine (II)

Desmethylimipramine (0.015 mmol; 4 mg) was dissolved in 0.2 ml of a dioxane-water (9 + 1) mixture, and 51.7 mg 10 g · l^{-1} PdO/$BaSO_4$ was added. The reaction mixture was stirred for 2 h with 4.7 ml carrier-free tritium. Preparative TLC (S_5) yielded a product with a radiochemical purity of 97% (TLC — $S_{4,5}$) and a specific activity of 2.4 TBq/mmol (65 Ci/mmol); the chemical yield was 62%.

[^3H]Imipramine (III)

[^{3}H]Desmethylimipramine (0.0065 mmol; 1.7 mg) was dissolved in a mixture of methanol (0.2 ml) and formaldehyde (0.02 ml) with 7.2 mg 10 g · l^{-1} PdO/$BaSO_4$ as catalyst. The reaction mixture was stirred for 2 h with 2 ml carrier-free tritium. Preparative TLC (S_4) yielded a product of a 96% radiochemical purity (TLC — $S_{4,6,7}$) and a specific activity of 2.9 TBq/mmol (78 Ci/mmol); the chemical yield was 60%.

Preparation of ^{125}I-labelled desmethylimipramine derivatives (VI, VIII)

Desmethylimipraminyl-N-succinyltyrosine methylester (V)

Dicyclohexylcarbodiimide (18 mg) in 2 ml methylene chloride was added to desmethylimipraminyl-N-hemisuccinate (IV) (0.06 mmol; 24 mg) and the mixture was stirred for 15 minutes. Then tyrosine methylester (0.08 mmol; 16 mg) in 4 ml methylene chloride was added and the mixture was stirred at room temperature overnight. The reaction mixture not containing free hemisuccinate was evaporated to dryness, then dissolved in 4 ml methylene chloride. The released dicyclohexylurea was filtered off. This process was repeated three times. The crude product was purified by chromatography on silica gel (S_8) to yield 24.4 mg i.e. 70% of pure product.

Desmethylimipraminyl-N-[3-(4-hydroxyphenyl)]propionamide (VII)

A solution of N-hydroxysuccinimide ester of 3-(4-hydroxyphenyl)propionic acid (0.1 mmol; 26 mg) in 2 ml dioxane was added to a solution of desmethylimipramine (0.08 mmol; 21 mg) in 5 ml dioxane and the reaction mixture was stirred overnight. The crude product obtained by evaporation was purified on silica gel (S_8). The yield was 25 mg (81%). Iodination of desmethylimipraminyl-N-succinyltyrosine methylester and desmethylimipraminyl-N-[3-(4-hydroxyphenyl)]propionamide was carried out according to *Hunter & Greenwood* (7) with a 30% radiochemical yield referred to $Na^{125}I$.

The schemes of preparation of radioligands labelled with ^3H or ^{125}I are shown in figure 1.

Determination in biological materials

It is not necessary to deproteinize plasma, serum or other biological fluids for this determination. Tissues after homogenisation, or subcellular fractions and biomembranes (100 µl) were made alkaline with 10 µl of 0.1 mol/l NaOH and extracted for 5 min with 0.5 ml heptane containing 3 g · l^{-1} isoamylalcohol in polyethylene tubes. The tubes were centrifuged for 5 min at 12000 *g*. Aliquots of 250 µl of the heptane phase were transferred into polyethylene tubes and the samples were extracted into 100 µl of 0.01 mol/l HCl. An aliquot (50 µl) of the water phase was added to 50 µl of 100 mmol · l^{-1} phosphate buffer pH 7.5. For determination in plasma, where various amounts of plasma are used depending on their presumed concentration, it is necessary to prepare a calibration curve with the same amount of control plasma taken from a subject who has not been given any drugs.

A 100 µl standard containing 25—1600 pg of drug, or the measured sample, was added to 100 µl ^3H-labelled ligand (0.3 pmol per sample) or ^{125}I-labelled ligand (2 fmol per sample) in 50 mmol · l^{-1} phosphate buffer pH 7.5 containing 0.1 g · l^{-1} sodium azide and 0.1 g · l^{-1} gelatine. When serum or plasma

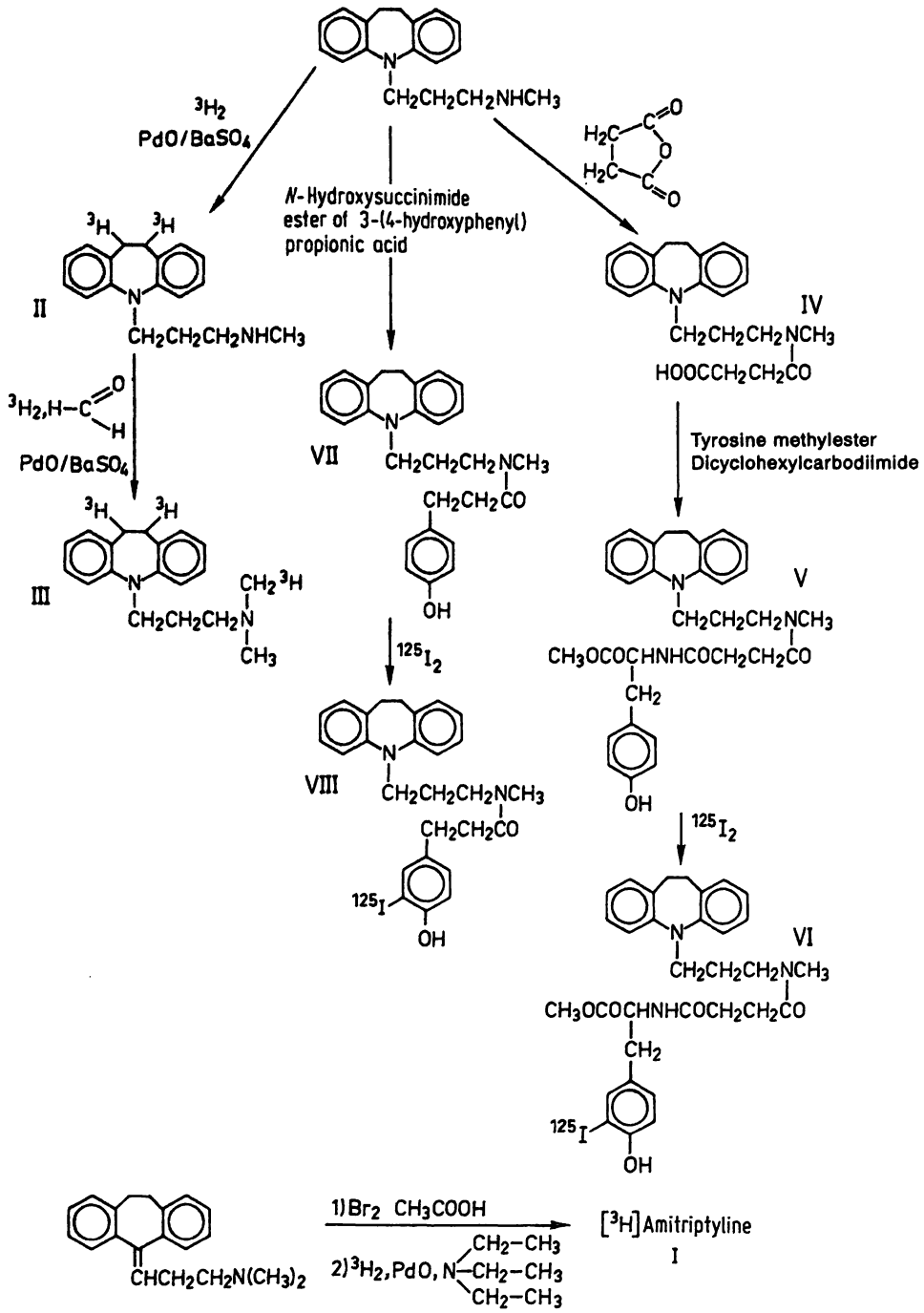

Fig. 1. Scheme of radioligand preparation.

are used, the standard curve is prepared with the same amount of control plasma used in the samples supplemented with 100 µl diluted antiserum. The antiserum was diluted 1 : 1000 with phosphate buffer containing sodium azide and gelatine. The samples were incubated for 2 h at 23 °C and then, in the case of ^{3}H-labelled ligand, 0.5 ml active charcoal Norit A (0.3 g · l^{-1} suspension in 50 mmol · l^{-1} phosphate buffer pH 7.5) was added. When the ligand labelled with ^{125}I was used, 23 g · l^{-1} polyethyleneglycol 6000 in 50 mmol · l^{-1} phosphate buffer pH 5.5 containing 0.2 g · l^{-1} bovine γ-globulin and 0.1 g · l^{-1} sodium azide were also added. After 20 min incubation at 0–4 °C the samples were centrifuged. Supernatant (400 µl) was taken from the samples labelled with ^{3}H and its radioactivity was measured in scintillation solution for 5 min. In the case of samples labelled with ^{125}I the precipitates were measured on a gamma counter for 2 min.

The measurements were carried out in triplicate. All results are expressed as means ± S.D.

Results

Standard curves for amitriptyline and imipramine are shown in the range of 50–6400 pg per sample (fig. 2). Standard curves for nortriptyline and [^{125}I]desmethylimipraminyl-N-succinyltyrosine methylester and [^{125}I] desmethylimipraminyl-N-[3-(4-hydroxyphenyl)] propionamide are given in figure 3. Cross reactions of various psychotropic drugs and their metabolites are given in table 1.

The results show the specificity of the reaction. Demethylated derivatives of tricyclic antidepressants have practically the same reaction and hydroxyderivatives have approximately ½ of the effect. Pheno-

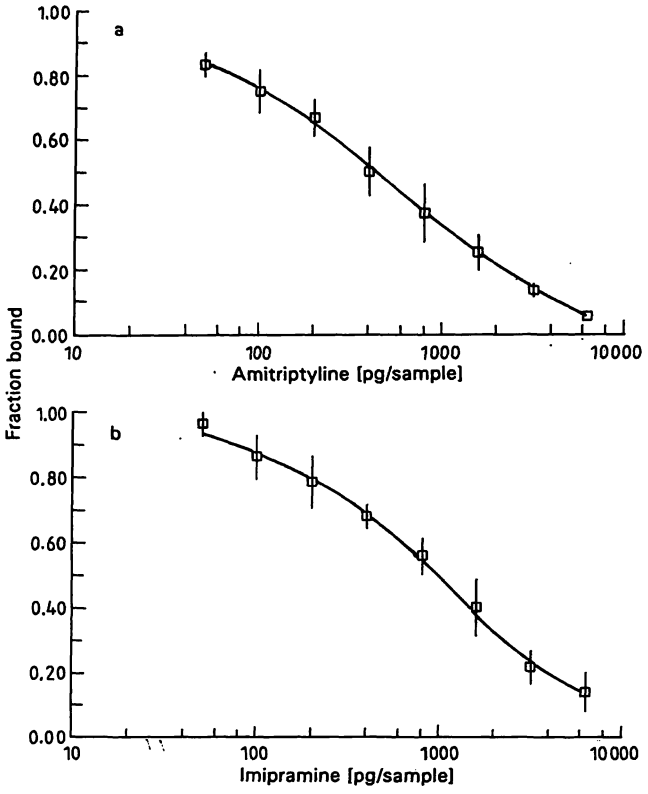

Fig. 2. Standard curve for a) amitriptyline and b) imipramine.

Tab. 1. Cross reaction of various tricyclic antidepressant drugs and structurally related compounds.

Compound	% Cross reactivity Antisera	
	Dibenz-azepines [^{3}H]imip-ramine	Dibenzcyclo-hexano-dienes [^{3}H]ami-triptyline
Imipramine	100	91
Amitriptyline	37	100
Chlorimipramine	55	44
Desmethylimipramine	94	86
Didesmethylimipramine	81	73
2-Hydroxyimipramine	61	59
2-Hydroxydesmethyl-imipramine	56	75
2-Hydroxyiminodibenzyl	0	0
2-Nitroimipramine	2	6
Prothiaden	92	97
Thioridazin	0	0
Chlorprothixen	15	22
Chlorpromazin	12	17

Cross reactivity was determined on a molar basis. Measurements were carried out in triplicate and the experiments were repeated 3 or 4 times. Standard deviation did not exceed 10% of the mean.

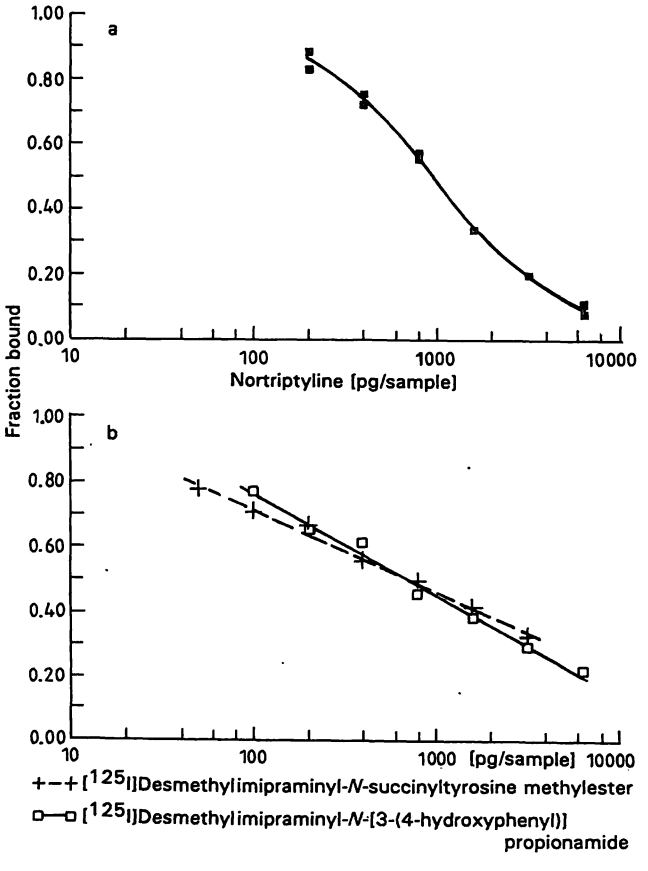

Fig. 3. Standard curve for a) nortriptyline and b) [^{125}I]desmethylimipraminyl-N-succinyltyrosine methylester (—+—) and [^{125}I]desmethylimipraminyl-N-[3-(4-hydroxyphenyl)]propionamide (—□—). The measurements were carried out in triplicate.

thiazine and thioxanthene derivatives show a 10—20% interference. High affinity was found in prothiaden [11-(3-dimethylaminopropylidene)-6,11-dihydrodibenzo(b,e)thiazepine], a substance structurally similar to imipramine. This method could also be used to determine this substance in biological tissues. The sensitivity of the method is high enough to detect concentrations lower than 1 $\mu g \cdot l^{-1}$. Error of the method is 5.3% $\pm$ 0.51 (n = 17) and recovery is 101.3 $\pm$ 5.94% (n = 19).

The method was tested in the determination of tricyclic antidepressants in patients treated with amitriptyline. Determinations were carried out 30 and 60 minutes after a single oral dose of 100 mg of amitriptyline and further at 1-hour intervals up to 5 h after the blood collection. Blood plasma separation was carried out immediately after the blood had been drawn and, again after 2-h standing at 4 °C. Neither the plasma levels nor the scatter of data were influenced by the time of keeping the sample (fig. 4). Dissociation of imipramine bound to erythrocyte membranes was studied both with [^{3}H]imipramine and by using the RIA method (fig. 5), and the results showed satisfactory agreement.

Another study showed that after a single dose of 100 mg amitriptyline the values in erythrocytes were half those found in plasma (fig. 6). After 7 days the plasma

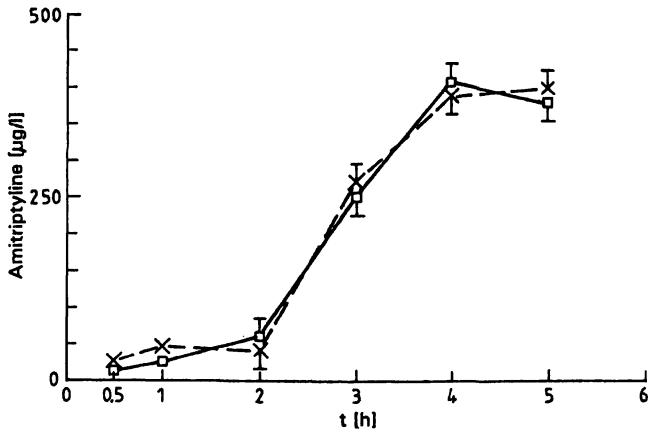

Fig. 4. Amitriptyline plasma levels after a single dose of 100 mg. The concentrations were determined in plasma immediately after its separation from the erythrocyte (—□—) and after a 2-hours standing at 4 °C (– – × – –).

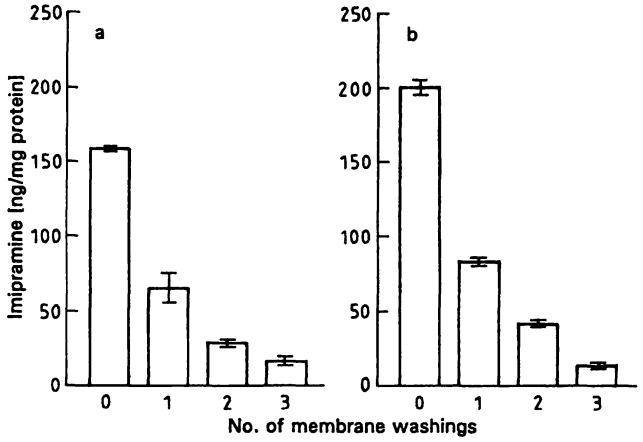

Fig. 5. Rate of dissociation of imipramine bound to erythrocyte membranes.
The determination was carried out using a) [^{3}H]imipramine and b) RIA with the same concentration of bound imipramine. Erythrocyte membranes were incubated with imipramine hydrochloride (1 µmol/l) in ice-cold buffered solution (50 mmol/l Tris, 120 mmol/l NaCl, 5 mmol/l KCl, pH 7.4) for 60 min. The membranes were repeatedly washed and centrifuged (40000 *g*, 10 min). Imipramine was extracted (see Methods) and determined by RIA.
The same method was used with addition of 3 nmol/l [^{3}H]imipramine and radioactivity was measured.

values were lower than the detection limit of the method, while the values in erythrocytes were 40 ± 18 µg · l^{-1} after 7 days and 26 ± 8 µg · l^{-1} after 14 days (mean ± S.E.M., n = 5). We concluded that it takes 21 days to eliminate the drug.

Discussion

A number of methods have been used for tricyclic antidepressant determination, including colorimetry, chromatography as well as radioisotopic and radioimmunological methods. The latter two methods are

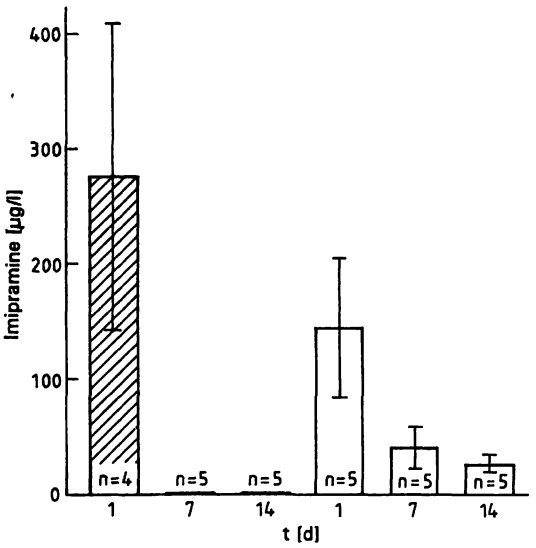

Fig. 6. Imipramine concentrations in plasma (▨) and erythrocytes (□) after a single dose of 100 mg.

found to be advantageous on account of their sensitivity and easy performance. However, there is the disadvantage that some metabolites may interfere and distort the results.

To prepare antisera we used the methods of *Brunswick* et al. (4) and *Hubbard* et al. (3). Desmethylimipramine and nortriptyline were converted into succinates which were subsequently conjugated with albumin and used for immunizing rabbits, i.e. antisera were raised against both dibenzazepine and dibenzcycloheptanodiene derivatives. For this reason, we labelled imipramine, desmethylimipramine and amitriptyline with tritium. The specific activities were very high, 2.9 TBq/mmol for imipramine, 2.4 TBq/mmol for desmethylimipramine and 4.3 TBq/mmol for amitriptyline, which contributed to the high sensitivity of the method. Despite the high specific activity of [^{125}]desmethylimipraminyl-N-[3-3′-iodo(4′-hydroxyphenyl)] propionamide in excess of 37 TBq/mmol, a higher sensitivity was not achieved, owing to changes in the side chain. The specificity of the method is good but demethylated metabolites, representing the main metabolites in man, which are also therapeutically effective, are determined at the same time.

While demethylated derivatives of tricyclic antidepressants affect primarily the catecholamine uptake, methylated derivatives exert their effect predominantly on indolamine uptake. It is presumed that the transport mechanisms for 5-hydroxytryptamine and noradrenaline are directly linked to tricyclic antidepressant binding sites, where they function as modulators. This was why we measured the tricyclic antidepressant levels in plasma and in cell membranes

after administering these drugs to man. The method was found to be highly suitable in view of its high sensitivity. The first methods developed in this field were significantly less sensitive (8). The results show that plasma levels drop to zero earlier than they do in cells. Dissociation of these substances from the cell membrane is slow, which has also been documented by the results obtained from repeated membrane washing in a large amount of buffer. We can conclude that after repeated administration of tricyclic antidepressants the drug remains in the cell membrane for a long period and can significantly influence the binding capacities of these substances.

Tricyclic antidepressants and their metabolites are determined by this method in the ratio of their affinities to antisera. With regard to the fact that the therapeutic effect of desmethylderivatives is comparable to that of the initial substance, their parallel determination shows the rate of therapeutic effect. The values of interferences in binding studies are also quite similar. After a single i. p. dose of amitriptyline to rats the ratio of plasma levels of amitriptyline and nortriptyline is about 4 : 1. The same occurs in the brain, although the concentrations of amitriptyline and nortriptyline are about 10 times higher compared with the values in plasma (9). Nortriptyline levels after chronic administration are about twice as high (compared with single dose administration) as determined by HPLC method. After administration of imipramine, *Nagy & Johansson* (10) determined imipramine and desmethylimipramine values in plasma and blood cells using a thin-layer chromatography method with densitometric evaluation. They found very similar levels of both substances in blood and blood cells after a single oral dose. However, they reported big interindividual differences. In the studies of *Suranyi-Cadotte* et al. (11) who investigated both [^{3}H]imipramine binding in platelets and imipramine and desmethylimipramine values in plasma, very similar levels of both substances were found after repeated administration of imipramine. The metabolism of tricyclic antidepressants, however, is significantly dependent on a number of factors. Stress also plays a special role in this respect (12).

Our method is highly sensitive for the direct determination of dibenzazepines and dibenzcycloheptanodienes in body fluids. The method can be used to determine tricyclic antidepressants in cell membranes and in tissues after extraction of these drugs as described.

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